Diagnostics of diarrheagenic *Escherichia coli* and *Shigella* spp. 1 2 JAN 2006

Field of invention

The present invention relates to a novel diagnostic assay for the detection of diarrheagenic *E.*
5   *coli* (DEC) by identification of specific genetic markers, e.g. by use of multiplex PCR. The
method further allows the evaluation of the pathogenic potential, which is valuable in relation
to the treatment of a patient. The method will be useful for the analysis of any material from
where alive bacteria can be generated, or from where bacterial DNA can be extracted. The
specific PCR product can be detected by a number of technologies that are faster and both
10   more sensitive and specific than conventional electrophoresis. The invention also includes a
method for the subtyping of a number the *E. coli* virulence genes that are believed to be
important in the treatment and epidemiological surveillance of diarrheagenic *E. coli*
infections.

15   General background

Diarrheagenic *E. coli* (DEC) strains isolated from intestinal diseases have been grouped into
at least six different categories based on epidemiological evidence, phenotypic traits, clinical
features of the disease they produce, and specific virulence factors. The currently recognized
categories of diarrheagenic *E. coli* include: Attaching and effacing *E. coli* (A/EEC) including
20   Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli*
(EIEC), Enteroaggregative *E. coli* (EAggEC), diffusely adherent *E. coli* (DAEC), and Shiga
toxin-producing *E. coli* (STEC), which are also referred to as Verocytotoxin-producing *E.*
coli (VTEC).

Table 1. Showing groups of diarrheagenic *E. coli* and characteristic virulence genes.

E. coli group	Positive for gene(s)	Corresponding toxin	Comments
VTEC	*vtx1* and/or *vtx2*	VT1 and/or VT2	May contain *eae* and/or *ehxA*
A/EEC	*eae*	Eae	Negative for any toxin genes and not belonging to the classical EPEC O:H serotypes.
EPEC	*eae*	Eae	Belong to classical O:H serotypes. Typical EPEC O:H serotypes are *bfpA*-positive, atypical EPEC are *bfpA*-negative. VTEC related EPEC strains may contain *ehxA*.
ETEC	*sta and/or elt*	ST and/or LT	
EIEC	*ipaH*	IpaH	

The most important groups are EPEC, ETEC, EIEC and VTEC whereas the role of EAggEC and DAEC are still being questioned. The definitions of these groups are not definitive and related to a number of genotypic- and phenotypic methods of characterization.

5   A definition adopted in 1995 identified the most important characteristics of EPEC as its ability to cause attaching and effacing (A/E) histopathology and its inability to produce Verocytotoxins. Typical EPEC of human origin possess a virulence plasmid known as the EAF (EPEC adherence factor) plasmid that encodes localized adherence on cultured epithelial cells mediated by the Bundle Forming Pilus (BFP), while atypical EPEC do not

10   posses this plasmid. The majority of typical EPEC strains fall into certain well-recognized O:H serotypes (10). According to this definition, the basic difference between typical and atypical EPEC is the presence of the EAF plasmid encoding BFP in the first group of organisms and its absence in the second. The definition is not static and may be changed as new types are discovered and described. The EPEC O:H serotypes that are currently regarded

15   as classical and newly recognised EPEC O:H serotypes by The International *Escherichia* and *Klebsiella* Centre (WHO) are shown in table 2.

Table 2. O:H serotypes regarded as classical and newly recognised EPEC O:H serotypes

O group	H antigen [a]	Comments
O26	H⁻; H11	O26:H⁻ and O26:H11 may also be STEC/VTEC
O55	H⁻; H6; H7	O55:H7, H10 and H⁻ may also be STEC/VTEC
O86	H⁻; H 8; H34	O86:H- may also be EAggEC H8 is a new type
O88	H-; H25	New type
O103	H2	
O111	H⁻; H2; H7	O111:H⁻ may also be STEC/VTEC or EaggEC
O114	H⁻; H2	
O119	H⁻; H2; H6	
O125ac	H⁻; H6	O125 may also be EaggEC
O126	H⁻; H2; H21; H27	
O127	H⁻; H6; H9; H21; H40	
O128ab	H⁻; H2; H7; H12	O128:H2 may also be STEC/VTEC
O142	H⁻; H6; H34	
O145	H-; H45	New type
O157	H-; H8; H16; H45	New types
O158	H⁻; H23	

[a] Non motile strains of *E. coli* are regarded as descendants of motile strains that have lost their

20   motility by mutation(s).

Apart from the well-recognized classical O:H EPEC serotypes, a large group of non-classical A/EEC serotypes of *E. coli* strains are found to be positive for the *eae*-gene. Together with EPEC, this group is referred to as Attaching and Effacing *E. coli* (A/EEC) based on the presence of the *eae*-gene and absence of toxin- or invasion genes. Like EPEC, they may be

5   positive or negative for the EAF plasmid but they may also be positive for the *ehx*A plasmid found in many VTEC strains, see below.

ETEC strains do not invade epithelial cells but produce one or more enterotoxins that are either heat-labile (LT), which is closely related to cholera toxin, or heat-stable (ST).

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EIEC are very similar to *Shigella*. Like *Shigella*, they are capable of invading and multiplying in the intestinal epithelial cells of the distal large bowel in humans. Genes involved in the invasive phenotype of EIEC and most *Shigella* spp. are carried on a 140 MDa plasmid designated pInv. Prominent among these virulence genes is a type III secretion

15   system (18). Also characteristic for the invasive phenotype is the *ipaH* gene, which is present in several copies on both the chromosome and the plasmid, making it especially suited as a diagnostic marker for EIEC and *Shigella* spp. (27).

VTEC strains are characterized by their ability to produce either one or both of at least two

20   antigenetically distinct, usually bacteriophage-mediated cytotoxins referred to as Stx1 or VT1 (first described as Shiga-like toxin I, SLTI) and Stx2 or VT2 (first described as Shiga-like toxin II, SLTII). Whereas STEC/VTEC refers to all *E. coli* strains that produce Stx/VT in culture supernatants (14,15), the term enterohemorrhagic *E. coli* (EHEC) has been used to refer to strains that have the same clinical and pathogenic features associated with the

25   prototype organism *E. coli* O157:H7 (16). In practice, EHEC is used to describe a subgroup of STEC/VTEC that causes hemorrhagic colitis (HC). Almost all STEC/VTEC O157:H7 strains harbour a large 60-65 MDa plasmid (9), designated pO157, which plays a role in the virulence(11). The large plasmid of O157 encodes the EHEC-hemolysin (Ehx), which is homologous to the *E. coli* α-hemolysin (20,21). A role for Ehx in the pathogenesis of

30   diarrhoeal disease has not been demonstrated but *ehxA* positive VTEC strains have been found more often in patients with Hemolytic Uremic Syndrome (HUS) than in patients with diarrhoea (6) and, together with the *eae*-gene in VTEC strains, serve as a predictor for more

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serious complications. O26:H11 strains also possess at least one plasmid in the range of 55-70 MDa and other O:H serotypes show a notable similarity with the large plasmids in O157 and O26 strains (16).

5   The diagnosis of DEC began in 1945 when Bray demonstrated the relation between *Bacterium coli var. neapolitanum* and diarrhoea in humans (3). A few years later Bray and Beaven used slide agglutination to type 95% of the bacteria isolated from stool cultures from children with diarrhoea (4). The breakthrough in typing was achieved in 1950, when the *E. coli* serotyping scheme was developed by Kauffmann (12). During the 1950s several new serogroups were
10   added to the list of those epidemiologically incriminated as causing diarrhoea (30). Meanwhile the enterotoxins of enterotoxigenic *E. coli* (ETEC) and the invasive properties of enteroinvasive *E. coli* (EIEC) had been described. Methods for detection included an infant mouse assay for the detection of ST, cell assays for LT, and inoculation of the eye of Guinea Pigs and subsequent development of keratoconjunctivitis for the detection of EIEC. In 1977, Konowalchuk *et al.* (15)
15   discovered a cytopathic effect in Vero cells from culture filtrates of *E. coli*. The effect could only be seen in Vero cells and not in Y1 mouse adrenal cells and Chinese hamster ovary (CHO) cells, and it was distinctly different from that of heat-labile enterotoxin. The cytotoxic effect was caused by one or more cytotoxins referred to as Vero toxins (VT) or Verocytotoxins (13).

20   Because of the above mentioned type diversity, rapid and easy methods of moderate cost for reliable identification and isolation of DEC strains independent of their serotype are required. A number of suitable methods for this purpose have been developed for each of the types but there is no internationally recognised standard procedure. These methods include biological assays, immunological methods, nucleic acid based assays or phenotypical tests such as O
25   grouping of commonly occurring DEC serotypes, enterohaemolysin production of the majority of VTEC types or the failure to ferment sorbitol or produce β-glucuronidase by most VTEC O157, and culture methods.

Unfortunately, these screening methods are incomplete because they are only directed against
30   a subset of the DEC strains.

The diagnosis of DEC have important implications for the evaluation of possible intervention during the course of illness. Prolonged diarrhoea caused by EPEC and A/EEC especially in children may require antibiotic treatment of the patient whereas treatment of patients with a VTEC infection is not recommended due to the possible increased risk of a more severe

5   outcome. In many countries, patients with a VTEC infection are quarantined or otherwise isolated due to the risk of contaminating other people. ETEC diarrhoea is not a very serious disease and usually self-limiting. It therefore usually does not require treatment. As is the case for VTEC infections, EIEC and *Shigella* infections are often succeeded by both quarantine and antibiotic treatment due to the low infectious dose and the risk of

10   contamination other people.

Pass, M. A. et al. (2001) (19) have published a method for detection of pathogenic *E. coli* in cultured faeces. PCR was used to amplify specific fragments in the genes encoding the following 11 virulence factors: VT1, VT2, VT2e, CNF1, CNF2, LTI, STI, STII, EaeA, Einv

15   and Eagg. It is stated that 4 multiplex-PCR combinations of primers gave adequate amplification of their respective genes. However, when the combination of multiplex-PCR with VT1, VT2, VT2e, EaeA, CNF2, Einv, LT and ST is shown, VT2e and VT1 are not visualised on the gel. This is an accepted fact that is explained in the article. Furthermore, the assay does not include a positive control.

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Patent Application WO9848046 describes a PCR assay that detects EHEC, ETEC, EPEC, EIEC and EAggEC that are specifically designed for real-time PCR analyses. However, real-time PCR is presently limited to 4 simultaneous genes per reaction because of the fluorophore overlap.

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López-Saucedo, C et al (2003) (17) are describing a method where the following 7 genes are detected in the same multiplex-PCR: *elt, sta, bfpA, eaeA, vtx1,vtx2 and ial*. They are analysing the PCR products by size identification on agarose gel electrophoresis; the assay does not include the *ehxA* gene and does not have a positive control.

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Compared to prior art the present method contains the following advantages:

- the use of *ipaH* for the detection of EIEC and *Shigella* spp. is a good genetic marker for this group of bacteria, as the gene is present in several copies both on the pInv plasmid

5   and on the chromosome . The use of *ial* is a poor diagnostic marker for these bacteria because it is only present on the plasmid, which is easily lost both *in vivo* and *in vitro*.

- The use of *ehxA* as a diagnostic marker allows a further estimation of the pathogenic potential giving rise to serious diseases, which is not possible by any of the prior art.

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- The use of 16S rDNA as positive control and the UNG system makes this method suitable as a reliable method for routine diagnostics. None of the prior art contains such considerations.

15   - The present method contains thoroughly validated tests both with respect to sensitivity and specificity (see example 1).

EPEC plasmids encoding the *bfpA*-gene and EHEC plasmids encoding the *ehxA*-gene have not been found together in the same strain and the two genes may therefore serve as useful

20   genotypic markers for the presumptive categorization of any *eae*-positive *E. coli* as either belonging to the A/EEC - EPEC group or to the EHEC group.

The method disclosed in the present invention detects the clinically relevant DEC types simultaneously and has very few limitations. The main advantage of this invention is that

25   subsequent to the identification of positive stool cultures, procedures for further analysis by supplementary PCR of the bacterial lysates obtained during screening are possible and could include: virulence gene subtyping by PCR followed by restriction digests or sequencing, O:H serotyping by sequencing of PCR amplified bacterial antigens, or other genotyping by for example microarray analyses. The procedure also allows for the referral of the lysate to more

30   specialised reference laboratories, which – in times where bioterrorism is ever threatening - will be safe and easy to understand for everybody at the primary screening laboratory facility. All primers chosen in the present invention were designed to match the most conserved

regions within the relevant genes. By doing so, the method is optimised to detect any possible subtype of the relevant genes, including new genetic subtypes that are expected to contain genetic changes in the less conserved regions, increasing the chance of being detected by the present method. However, as for any PCR based method, it requires continuously updating

5   and validation whenever new genotypes are being described. As our laboratory serves as The International *Escherichia* and *Klebsiella* Centre (WHO) there will be no problem in obtaining presumptive new types.

10   **Summary of the invention**

The presently preferred embodiments of the present invention are outlined in the following points:

1) Novel multiplex-PCR combination detecting the 8 genes *bfpA, ehxA, vtx1, vtx2, eaeA, ipaH, sta* and *elt,* which is found to be the most suited gene combination for the

15   characterization of diarrheagenic *E. coli.*, and including a PCR-control derived from 16S rDNA (positive control).

2) Intensive validated experimental procedure, showing superior sensitivity and specificity compared to other publications.

3) Descriptions of how the multiplex-PCR can be combined with other technologies in

20   order to decrease time of analysis and improve sensitivity.

4) Routine diagnostic consideration with respect to carry-over prevention, by the use of the UNG system.

5) Protocols for the subtyping of the virulence genes: *eae, vt1 and vt2* by either, direct sequencing of the amplicons generated in the multiplex-PCR, or by sequencing of a

25   larger fragment generated by a new PCR.

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Detailed description of the invention

The present invention discloses a method for simultaneous detection of diarrheagenic
5   *Shigella spp.* and *E. coli* (DEC) including A/EEC & EPEC, ETEC, VTEC, EIEC and
especially strains with the *ehxA* gene.

A preferred embodiment of the invention detects the presence of the genes *ehxA, eae, vtx1,
vtx2, ipaH, sta, elt and *bfpA* and is able to detect *Shigella spp.* by the presence of the *ipaH*
10   gene. The presence of the genes can be detection of the genes themselves or parts hereof,
RNA or polypeptides coded by the genes.

The screening method can be performed by nucleotide sequence amplification technique,
such as PCR, multiplex PCR, real-time PCR, most preferably multiplex PCR, with a selected
15   set of primers and incorporating a positive control using 16S rDNA. Possible contamination
of samples is preferably reduced by incorporating the UNG system.

Detection of the genes can be performed by size identification, e.g. by agarose gel
electrophoresis or capillary electrophoresis or with a hybridisation probe.
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The sample material to be analysed can be any material from where bacteria can be extracted,
e.g. stool samples, consumables etc.

The screening method can be used as an *in vitro* diagnostic method for determining the risk
25   of being infected with a pathogenic organism which gives rise to haemolytic uremic
syndrome (HUS) or hemorrhagic colitis, by detecting the *ehxA* gene in the sample.

The invention discloses a specific set of primers and probes for the respective genes but are
not restricted to these.
30

The invention also discloses a kit for the screening, which comprises, in a single or in
separate containers, nucleotide sequences which are able to prime amplification in a

nucleotide sequence amplification reaction, such as PCR, of the genes: *ipaH, eae, ehxA*, and *sta*, or parts of these genes or the complementary strands to the genes or parts thereof. Additionally the kit can comprise nucleotide sequences which are able to prime amplification of the genes: *vtx1, vtx2, elt,* and *bfpA*, or parts of these genes or the complementary strands to

5   the genes or parts thereof. The described kit can also contain nucleotide sequences which are able to hybridise (preferably under stringent conditions) with the genes: *ipaH, eae, ehxA, sta, vtx1, vtx2, elt,* and *bfpA* or parts of these genes or the complementary strands to the genes or parts thereof. Preferably the kit comprises a means for a control, such as primers for 16S rDNA.

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Definitions and abbreviations:

A/E: <u>a</u>ttaching and <u>e</u>ffacing

A/EEC: <u>a</u>ttaching and <u>e</u>ffacing <u>E</u>. <u>co</u>li

Amplicon: *syn.* "PCR product"

bfpA: <u>b</u>undle <u>f</u>orming <u>p</u>ilus, structural gene, subunit A; Virulence factor in EPEC, which is involved in the initial adherence of the bacteria to the intestinal cells.

bp: <u>b</u>ase <u>p</u>air

Capillary electrophoresis: Capillary electrophoresis (CE), is a technique where an electrophoretic separation takes place in a thin capillary tube filled with buffer. A sample is injected at one end, either by electrophoresis or by pressure, and an electric field of 100 to 700 volts/centimeter is applied across the capillary. It is generally used for separating ions, which move at different speeds depending on their size and charge, when the voltage is applied. At the end of the capillary each of the separated analytes are measured by a detector in a time dependent manner. CE is usually run with an internal standard that allows size determination of the separated sample molecules.

Carry-over prevention: dUTP is incorporated in all PCR products instead of dTTP. Before PCRs

are subjected to thermocycling, they are incubated with UNG (uracil-DNA glycosylase) that degrades any single or double stranded DNA containing dUTP, but has no effect on dTTP containing DNA. By this procedure, possible contamination from other PCRs is reduced, while the amplification of bacterial DNA is unaffected.

DAEC: diffusely adherent *E. coli*

DEC: diarrheagenic *E. coli*

eae: *E. coli* attaching and effacing: intimin. Virulence factor from EPEC, A/EEC or VTEC.

EAF: EPEC Adherence Factor plasmid. Plasmid containing BFP

EaggEC: enteroaggregative *E. coli*, syn. EAEC

EHEC: enterohemorrhagic *E. coli*

ehxA: enterohemolysin, structural gene, subunit A

EIEC: enteroinvasive *E. coli*

elt: gene encoding heat labile enterotoxin (LT)

EPEC: enteropathogenic *E. coli*

ETEC: enterotoxigenic *E. coli*

estA: alternative name for *sta*; the gene encoding heat stable enterotoxin (ST)

estA-human: the humane variant of *estA* (*sta*)

estA-porcine: the porcine variant of *estA* (*sta*)

GI: gastrointestinal tract

HC: hemorrhagic colitis

HUS: haemolytic uremic syndrom

ipaH: invasive plasmid antigen H

LT: heat-labile enterotoxin

Luminex technology: microbeads of different internal colors are labeled with hybridization probes representing different genes. These beads are hybridized with fluorescence labeled sample DNA (usually PCR products) under stringent condition. After the hybridization has taken place, the mixture is injected into the instrument that uses microfluidics to align the microbeads in a single file where lasers illuminate the colors inside and on the surface of each microbead. The optics capture the combination of color coded microbeads and hybridized sample molecule.

Microarray technology: hybridisation probes representing different genes are chemically linked to different spots on a solid surface, usually a small glass slide. Fluorescence labeled sample DNA (usually PCR products) are hybridised to the capture probes under stringent condition. After the post hybridization washing steps, only sample DNA with nucleotide sequences complementary to the capture probes will stay bound to the slide. Bound PCR products at specific spots of known capture probes, are registered by their fluorophore emission.

Multiplex PCR: PCR with more that one primer set present in the same reaction, where each primer set is amplifying a unique locus if the specific template is present.

O:H: specific serotype; "O" refers to the LPS O antigen, and H refers to flagellar antigen.

Real-time PCR: Detection of the PCR while the temperature cycling is still in progress. This can be done by the measurement of emitted fluorescence, which is linked to the reaction. The

fluorescence can originated from fluororphores that bind to the double stranded amplicons
(sequence unspecific interchelating agent, ex; SYBR Green), or it can originate from sequence
specific probes that are designed downstream of the primers. Such probes will emit light only when
digested by the polymernase because they contain a fluorophore and a corresponding quencher.
Real-time PCR is limited to 4 simultanous genes per reaction because of the fluorophore overlap.

stx 1 / 2: genes encoding verocytotoxin 1 / 2

ST: heat stable enterotoxin

sta: gene encoding heat stable enterotoxin (ST); today also called *estA*

STh: heat stable entertoxin (human), *syn.* STIB

STp: heat stable enterotoxin (porcine), *syn.* STIA

VT 1 / 2: verocytotoxin 1 / 2, *syn.* shiga like toxin (Stx)

VTEC: verocytotoxin producing *E. coli*

vtx 1 / 2: gene encoding verocytotoxin 1 / 2 (VT 1 / 2)

UNG: uracil-DNA glycosylase

Enclosed in the present invention is the possibility of performing diagnostic PCR, both on
DNA prepared directly from human faecal samples, or on DNA prepared from colonies
5   grown from faecal samples. Performing PCR on DNA purified directly from faeces is an
attractive strategy, as it saves time and labour. Besides that, direct PCR can detect dead cells,
and cells prone to loose plasmids during *in vitro* growth, and is not affected by the selectivity
that a growth step might introduce. However, it is also important to have a fast and easy way
to test plated out cell material, when single colonies need to be isolated. For that reason, the

present invention contains a method, which relies on simple boiling and centrifugation for preparing PCR-usable template DNA.

In the present invention, sensitivity limits are established by making a dilution series of
5   pathogenic bacteria in a background of non-pathogenic bacteria. That is the most important way to test the sensitivity, as this situation is closest to the compositions of clinical samples. In the present invention, a positive result can be obtained, if the template DNA has a composition, where one pathogenic bacterium is present among 10^4 non-pathogenic bacteria.

10   High specificity relies on good primer design, but is also depended on experimentally testing of the assay on different strains know to harbour different homologous of the relevant genes. The present invention has been tested 100% specific on 124 different reference strains obtained from The International *Esherichia* and *Klebiella* Centre, WHO, Statens Serum Institut, Denmark (see table 5).
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In a presently preferred embodiment, the present invention is using the genes coding for the following virulence factors: BfpA, EhxA, VT1, VT2, Eae, IpaH (same as Einv), ST and LT. Genes encoding both VT2,VT2c, VT2d and subtype VT2e will be amplified by the present
20   PCR, and result in a PCR product of the same size. Besides that, the present invention has included a universal primer-pair towards 16S rDNA as a positive control for the PCR. As for the combination of genes in the multiplex-reaction, the present invention is able to perform sensitive and specific PCR with all 8 virulence genes and 16S rDNA present in the same multiplex reaction.
25

The present invention includes all the relevant genes for the currently recognised and clinically important groups of DEC. The choice of genes allows for a rapid evaluation of the further treatment and interventional strategies in relation to the individual patient in order to minimise complications and the spread of highly pathogenic bacteria to contacts or the
30   environment.

The present invention contains a method for the subtyping of the virulence factors VT1, VT2, Eae and other genes. Subtyping of these virulence genes is increasingly being accepted as an

important part of characterizing VTEC infections, especially in relation to the proper treatment (5).

The present invention solves the diagnostic problem of screening for human pathogenic *E. coli* groups. The method relies on specific multiplex-PCR amplification of 8 virulence genes allowing a distinction between the pathogenic *E. coli* groups: ETEC, VTEC, A/EEC including EPEC and EIEC, and provides important distinction between typical/atypical EPEC strains and additional information on the presence or absence of the EHEC plasmid. The method is based on primers chosen to match all clinically relevant subtypes of the given virulence genes. The PCR setup is designed to enclose all primer sets in one single reaction, leading to the specific amplification of any given template present. The method was optimised to result in the best sensitivity and specificity. This was done by analysing DNA from 10-fold serial dilutions of bacterial colonies known to harbour different subtypes of the relevant virulence genes. The method therefore, allows for the analysis of any material from where alive colonies can be cultured. Of special interest is, the analysis of stool samples from diseased patients, where time, sensitivity and specificity are critical parameters in effective diagnoses. Also, the analysis of consumables has a high value as it may prohibit the spread and intake of contaminated foods. Due to the well characterized sensitivity limits, the methods also has the potential of analysing DNA purified directly from the primary sources.

The PCR primers, -probes, -reagents and -temperature conditions were optimised to perform well in combination with a number of different technologies. These technologies fall into two different groups

- DNA purification directly from the source. This can be done by a number of different commercial kits described in section 5 (Example 3-6 and 9).
- PCR products detection by either capillary electrophoresis, real time PCR or solid-face capture probe techniques like; membrane/ELISA hybridisation, DNA chips or Luminex ®.

The present invention also encloses a real-time PCR setup with optimised PCR conditions including specific primer and probe design. Besides that, the present invention also contains the option of amplifying all 9 genes in the same reaction, in a both sensitive and specific manner (non-real-time PCR setup). This is possible because the concentration of every

reagent has been carefully optimised (Example 8), and because the present invention (in this setup) is not burdened by the addition of a specific probe for every gene in the assay.

The present invention includes hybridisation probes specifically designed and optimised for
5   constituting the capture probes, in solid surface hybridisations like membrane hybridisation or hybridisation in microtiter plates, DNA microarrays and hybridisation on microbeads (ex. Luminex ® technology). Finally, the PCR products of the present invention lie within the size-range that should be easily detectable by capillary electrophoresis, which is faster, more sensitive and accurate than gel-electrophoresis.
10

Being able to use these technologies in combination with the multiplex PCR, results in a number of advantages compared to traditional diagnostics. Firstly, direct DNA purification from the source is not affected by the selectivity that a growth step might introduce, dead bacteria and bacteria that easily loose plasmids can be detected, and the entire procedure is
15   much faster. Secondly, due to the multiplex setup, it only requires one PCR to screen for the entire 8 virulence genes. Thirdly, the technologies used for amplicon detection are faster and more sensitive than traditional methods.

Specific primers are of major importance in a diagnostic PCR setup. The pivot of this
20   problem is sequence analysis of the available data in Gene Bank. The primer and probes were designed on basis of the considerations described below.

The heat stable enterotoxin (ST) of ETEC is a small monomeric protein of 18-19 amino acids encoded by the *sta* gene, of approximately 220 bp (18). The relative few submitted nucleotide
25   sequences of *sta*, available in Gene Bank, fall into a number of phylogenetic groups based on ClustalW comparisons. The groups of genes encoding heat stable enterotoxins were as follows: five *staI* genes referred to as *staI-human* (accession numbers: J03311, M34916, M29255, M18346 and M18345) made up their own cluster, two other *staI* genes referred to as *staI-porcine* (accession numbers: M25607 and M58746) were more related to the heat
30   stable enterotoxins from *Yersinia enterocolitica* and *Vibrio cholera*, and two separate clusters were made up of *staII* genes and the gene encoding EAST1 from enteroaggregative *E. coli* (EAggEC). As both *staI*-human and *staI-porcine* have been found in humans (18) both

variants were included in the PCR. But due the sequence diversity, separate primer pairs were designed for each variant, resulting in indistinguishable product size when analysed by agarose gel electrophoresis (151 and 160 bp). Primers were not designed towards the gene encoding STII or STb (accession number M35729 and AY028790) as these variants are

5   rarely found in humans (18). *staI* primers did not align with *Yersinia enterocolitica* heat stable enterotoxin (D63578), *Vibrio cholerae* heat-stable enterotoxin (M97591) or, EAST1 (AB042005), though they share some sequences similarities.

The heat labile enterotoxin (LT) of ETEC is composed of one A-subunit and 5 identical B-

10   subunits encoded by the *elt* gene. The toxin can be divided into LTI and LTII based on serology and host pathogenesis. The genes encoding the A- and B-subunits are 777bp and 375bp long, respectively (7). The cholera enterotoxin produced by *Vibrio cholerae* is about 75% identical in the nucleotide sequence to the LTI of *E. coli*. The sequences of subunit A from nine *eltI* genes (accession number: V00275, S60731, AF242417, AB011677, M35581,

15   M15261, K01995, M15362 and M57244) were compared to a number of *eltII* and *Vibrio cholerae ctx* genes. Due to the desired specificity towards *E. coli*, the clinical unimportance of *eltII* (18) and the relative low homology between *eltI* and *eltII* / *ctx*, probe and primers were designed to match *eltI* only, and result in an amplicon of 479 bp.

20   Intimin is encoded by the *eae* gene in either A/EEC (including EPEC) or VTEC, and has a size of approximately 2810 bp. Based on the divergent sequences in the last third of the 3-prime end, at least 8 subtypes can be identified. At least one of each subtype was present in the gene alignment and the following accession numbers were used: AF081186, AF253560, U60002, AB040740, AJ308552, AF116899, AF449419, AF081184, AJ308551, AF449416

25   and AJ298279. Probe and primes were deigned to match all tested gene sequences, and the PCR-product has a size of 377 bp.

The virulence factor, bundle forming pilus (Bfp) from EPEC, is encoded by an operon consisting of 14 genes, including the 580 bp structural gene *bfpA*. Based on sequence

30   comparisons, the *bfpA* genes fall into an alpha and a beta type. Probe and primers were deigned to match both types by aligning the genes with the following accession number:

AF304478, AF304486, AF304482, AB024946, AF304480, AF304477, AF304484, Z12295, AF382948. The resulting amplicon was 307 bp long.

Both vero toxin 1 and 2 (VT1 and VT2) from VTEC are composed of an A- and a 5 B-
5   subunits. The gene encoding the A-subunit is approximately 960 bp long, and the gene encoding the B-subunit is approximately 270 bp long. As the homology between VT1 and VT2 is relatively low (about 50% identity), and because of the desirable differentiation between the two toxins, specific probes and primers were designed to each gene.

10   Based on the nucleotide sequence it is difficult to distinguish between *vtx1* from *E. coli* and shiga toxin 1 from *Shigella* spp. Also, all *vtx1*-genes from *E. coli* share very high homology. Probe and primers were designed to match all *vtx1*- genes from *E. coli* and all shiga toxin 1 genes from *Shigella* spp, by alignment of the genes with the accession numbers: AF461172, AJ279086, AF153317, AJ132761, Z36899, AB030485, AB035142, AF461166, AJ251325,
15   AJ314839 and M19473. The resulting PCR product is 260 bp long.

Most *vtx2*-genes share relative high identity (above 90%). However, one group of genes seems to make a unique cluster, consisting of the *vtx2f* (accession numbers AJ270998 and Aj010730) and *vtx2va* (M29153) (now renamed *vtx2f*) subtypes, with about 60% identity to
20   the other *vtx2* genes. Due the relative low sequence homology, and the fact that most *vtx2f* and *vtx2va* are not found in humans (5), probe and primers were designed to match the major *vtx2* group, by aligning the *vtx2* sequences: AJ313015, AP000363, AB048228, L11078, X81415, X81418, X61283, M36727, AB017524, AF291819 and Y10775. The PCR product for VT2 was designed to be 420 bp long.
25

Enterohemolysin A (Ehx), often found in EHEC is encoded by the ca. 3000 bp long *ehxA* gene, which is part of the 4-gene enterohemolysin operon. The *ehxA* genes are a very homogenous group of genes, and probe and primers were designed to match all known subtypes. The following accession numbers were used in the gene alignment: X79839,
30   AB032930, AF074613, X86087, X94129, AB011549 and AF043471. The PCR product for *ehxA* was designed to be 530 bp long.

17

The invasive plasmid antigen *ipaH*-gene is carried in multiple copies on both the 140 MDa
invasive plasmid as well as on the chromosome of EIEC strains and *Shigella* spp. The
advantage of using this gene, rather than the *ial*-gene is that it remains detectable despite the
loss of the plasmid. Probe and primers were designed to match all genes analysed in the gene
5   alignment, made up of the following genes: AL391753, M32063, AF047365, M76445,
M76443, AF386526, AF348706 and M76444. The size of the PCR product is 647 bp.

16S rDNA was chosen as a positive control, because many Gram-negative bacteria found in
the human GI share high sequence homology. Thus, the detection of templates, that matches
10   the primers and probe is very high under any given clinical conditions (even in antibiotic
treated patients). Probe and primers were chosen to match as many as possible of the
common bacteria from the human GI. The 16S rDNA control band is 1062 bp long.

Primers were designed with the following parameters: 55-57°C melting temperature, GC-
15   content between 45-60%, length between 20-24 bp, lowest possible likelihood of dimer- and
hairpin formation, optimal entropy in the ends and distinguishable amplicon sizes. Each
primer set was tested individually under varying PCR conditions, and the optimum
conditions were used to construct the PCR conditions in the multiplex reaction. Primers were
redesigned until they met the desired level of sensitivity and specificity so all primer sets
20   were able to amplify any given target present in the sample. Probes were designed to have a
melting temperature between 65°C and 67°C, the least possibility of dimer- and hairpin
formation and no G in the 5'-end. The primer sets were optimised by testing the PCR
methods on a number of different strains under different template conditions.

25   The present invention also contains a method for subtyping the *vtx1, vtx2* and the *eae* genes.
By using one of the PCR primers in a sequencing reaction, performed on the PCR products,
the resulting sequence can by phylogenetically analysed and assigned to a specific subtype by
the comparison to sequences of known subtype. If the PCR product does not contain subtype
specific sequence, a set of PCR primers can be designed to amplify a larger fragment
30   containing more subtype specific sequences.

Table 3. Listed are the 8 virulence genes and 16S rDNA used in the multiplex-PCR assay, together with amplicon size, final primer concentration,and primersequences.

Virulence factor	E. coli group	Gene locus	Primer-name	Amplicon size (bp)	Primer conc. (µM)	Primer sequence (5'→ 3')
Heat labile entero-toxin I (LT1)	ETEC	elt	LT-F	479	0.4	AAACCGGCTTTG-TCAGATATGATG
			LT-R		0.4	TGTGCTCAGAT-TCTGGGTCTCC
Heat stable entero-toxin I (ST1)	ETEC	sta	ST-F	171	0.25	TCACCTTTCG-CTCAGGATGC
			ST-R		0.25	ATAGCACCCG-GTACAAGCAGG
		estA-humane	ST-Fh	151	0.4	TTTCGCTCAGGA TGCTAAACCAG
			ST-Rh		0.4	CAGGATTACAACA CAATTCACAGCAGTA
		estA-porcine	ST-Fp	160	0.4	CTTTCCCCTCTTTTAGT CAGTCAACTG
			ST-Rp		0.4	CAGGATTACAACAAAG TTCACAGCAG
Intimin (Eae)	EPEC / VTEC	eae	eae-F	377	0.2	GGYCAGCGTT-TTTTCCTTCCTG
			eae-R		0.2	TCGTCACCAR-AGGAATCGGAG
Shiga toxin 1 (Stx1) / Verocytotoxin 1 (VT1)	VTEC Shigella	stx1/ vtx1	VT1-F	260	0.25	GTTTGCAGTTG-ATGTCAGAGGGA
			VT1-R		0.25	CAACGAATGG-CGATTTATCTGC
Shiga toxin 2 (Stx 2) / Verocytotoxin 2 (VT2)	VTEC	stx2 / vxt2	VT2-F	420	0.25	GGAATGCAAATC-AGTCGTCACTC
			VT2-R		0.25	GCCTGTCGCCA-GTTATCTGACA
Invasion plasmid antigen H (ipaH)	EIEC / Shigella	ipaH	ipaH-F	647	0.1	TTGACCGCCT-TTCCGATACC
			ipaH-R		0.1	ATCCGCATCA-CCGCTCAGAC
Entero-hemolysin A (EhxA)	VTEC / EPEC	ehxA	ehx-F	533	0.05	GGGAAAAGCC-GGAACAGTTCT
			ehx-R		0.05	CCAGCATAAC-AGCCGATGTGAT
bundle-forming pilus A (BfpA)	EPEC	bfpA	bfp-F	307	0.4	TCCAATAAGKC-GCAGAATGCTA
			bfp-R		0.4	CACCGTAGCCT-TTCGCTGAAG
16S rDNA	most gram ÷	16S	16S-F	1062	0.25	GGAGGCAGCA-GTGGGGAATA
			16S-R		0.25	TGACGGGCGG-TGTGTACAAG

R = G or A, Y = C or T, K = G or T

The present invention contains PCR conditions that have been optimised to work with the carry-over prevention systems using dUTP and UNG. In order to prevent contamination from other PCRs, dUTP is incorporated into PCR products instead of dTTP. Before PCRs are subjected to thermocycling, they are treated with UNG that degrades dU-containing PCR

products, but has no effect on native template DNA. The same level of sensitivity and specificity as described above is obtained when UNG and dUTP are included in the assay.

5   The PCR products can be analysed by size identification on agarose gel electrophoresis as every PCR-product has a unique size. Besides that, the present invention encloses a number of faster and more sensitive solutions for identification of the PCR products. For each gene, a hybridisation probe has been designed, from a conserved region within the gene. This feature adds an extra level of specificity, as the PCR product must have the right internal sequence in order to be detected. The specific hybridisation probes can be utilized in a number of

10   different technologies. Firstly, real-time PCR can detect a positive PCR, before the thermocycling has run to completion. Besides the obvious time saving advantage, this technology is far more sensitive and specific than agarose gel electrophoresis. Real-time PCR is technically limited to a maximum of 4 genes per multiplex reaction, which means that the total of 9 genes needs to be analysed in 3 reactions. Secondly, the specific probes can

15   constitute the capture-probes on solid surfaces like nylon membranes, ELISA-plates or DNA microarrays, where a stringent hybridisation can take place, which is subsequently analysed due to colour-coded reagents. Thirdly, capture-probes can also be situated on microbeads (ex. Luminex ® technology), where the specific hybridization is analysed from the combination of colour-coded beads and colour-coded PCR-products. Finally, and not based on

20   hybridisation, PCR-products can be identified by capillary electrophoresis, which is faster, more sensitive and accurate than gel-electrophoresis.

Figure legends

25   **Fig. 1.** Two *E. coli* patogenic reference strains are mixed in equal volumes, and serially diluted in a constant background of a non-pathogenic E. coli strain (D2103). One pathogenic E. coli strain habours *vtx1*, eae, *vtx2* and *ehxA* (strain D2164), while the other pathogenic strain (fr1368) contains *ipaH*. All PCRs contain a total amount of DNA corresponding to the preparation of

30   approximately 0.05 bacterial colony.
Lane 1: only D2103,
Lane 2 only D2164 and fr1368,

Lane 3: 1/10 of D2164 and fr1368 relative to D2103,

Lane 4: $1/10^2$ of D2164 and fr1368 relative to D2103,

Lane 5: $1/10^3$ of D2164 and fr1368 relative to D2103,

Lane 6: $1/10^4$ of D2164 and fr1368 relative to D2103,

5   Lane 7: $1/10^5$ of D2164 and fr1368 relative to D2103 and

Lane 8: $1/10^6$ of D2164 and fr1368 relative to D2103

Fig. 2. Two E. coli pathogenic reference strains are mixed in equal volumes, and serially diluted in a constant background of a non-pathogenic E. coli strain

10   (D2103). One pathogenic E. coli strain habours *bfpA* and *eae* (strain D1826), while the other pathogenic strain contains *sta* and *elt* (strain D2168). All PCRs contain a total amount of DNA corresponding to the preparation of approximately 0.05 bacterial colony.

Lane 1: only D2103,

15   Lane 2 only D1826 and D2168,

Lane 3: 1/10 of D1826 and D2168 relative to D2103,

Lane 4: $1/10^2$ of D1826 and D2168 relative to D2103,

Lane 5: $1/10^3$ of D1826 and D2168 relative to D2103,

Lane 6: $1/10^4$ of D1826 and D2168 relative to D2103,

20   Lane 7: $1/10^5$ of D1826 and D2168 relative to D2103 and

Lane 8: $1/10^6$ of D1826 and D2168 relative to D2103

Fig. 3. PCR test of 8 different strains (lane 1-8). Four strains (lane 1, 2, 4, and 6) harbouring a combination of the 6 virulence genes: *sta, vtx1, eae, elt, ehxA*

25   and *ipaH*. Lane 3, 5, 7 and 8 were tested negative for pathogenic E. coli. Lane 9 contains 100 bp DNA marker. Marker to the left of lane 1 was removed for better visualization of gene designation, but was present when genes were assigned.

30   **Fig. 4.** PCR test of 8 different strains (lane 1-8) harbouring a combination of the 5 virulence genes: *vtx1, bfpA, eae, vtx2* and *ehxA*. Lane 9 contains 100 bp DNA marker. Marker to the left of lane 1 was removed for

better visualization of gene designation, but was present when genes were
assigned.

Examples

5   Examples 1-2 contains experimental data that shows how this method perform with respect
sensitivity and specificity, and how is can be applied as a diagnostic tool in a routine
laboratory. Examples 3-6 are intended to illustrate the invention, and describe how is can be
applied in combination with other technologies. The combination of the following steps: a)
DNA extraction; b) multiplex PCR and; c) method of PCR product detection, is not the

10   restricted to the ones mentioned in the examples, but will work in any preferred combination.
The specific method was first developed to diagnose human *E. coli* infections from the
bacterial presence in stool samples, but most of the DNA purification methods will work on a
variety of different starting materials. Example 7 deals exclusively with the genetic subtyping
of genes encoding either VT2 and/or eae from either VTEC or EPEC infections.

15

Contents:

Example 1:   - Template DNA prepared from plate grown cells by simple boiling procedure.

20   - Multiplex-PCR on the genes encoding the following *E. coli* virulence factors:
ST,   LT, Eae, BfpA, VT1, VT2, EhxA and IpaH.
               - Identification of PCR products by gel electrophoresis.
               - This example shows how the multiplex-PCR method performs with respect to
                 sensitivity and specificity.

25

Example 2:   - DNA extraction from bacterial colonies, derived from fecal samples by
growing   fecal samples overnight on agar plates.
               - Multiplex PCR on the genes encoding the following *E. coli* virulence factors:
ST,   LT, Eae, VT1, VT2, and IpaH.

30   - Identification of PCR products by gel electrophoresis.
               - This example shows how a method performs in a routine diagnostic laboratory
                 compared to a DNA hybridisation technique.

Example 3: - DNA purified directly from feces by performing cell lysis and separation
of magnetic beads that bind DNA (Kingfisher, Thermo Labsystems, Finland).
- Multiplex PCR on the genes encoding the following *E. coli* virulence factors:
5   ST, LT, Eae, BfpA, VT1, VT2, EhxA and IpaH.
- Detection of PCR products by LUMINEX® technology.
- This example describes a theoretical procedure for the above technologies.

Example 4: - DNA purified directly from feces, by separation of magnetic beads that bind
10   bacterial cells followed by cell lysis and ethanol wash (Genpoint A.S,
Norway)
- Multiplex PCR on the genes encoding the following *E. coli* virulence factors:
ST, LT, Eae, BfpA, VT1, VT2, EhxA and IpaH.
- Detection of PCR product by hybridisation to solid-phase capture-probes on
15   ex. nylon membrane, plastic surfaces or DNA chip/microarrays.
- This example describes a theoretical procedure for the above technologies.

Example 5: - DNA purified directly from feces, by DNA absorption/entrapment in fibrous
membranes (FTA Technology, Promega)
20   - Multiplex PCR on the genes encoding the following *E. coli* virulence factors:
ST,
LT, Eae, BfpA, VT1, VT2, EhxA and IpaH.
- Detection of PCR products by real-time PCR.
- This example describes a theoretical procedure for the above technologies.

25
Example 6: - DNA purified directly from feces by use of cell lysis, absorption and elution
of DNA from spin columns (ex. QIAamp® DNA Stool Mini Kit, QIAGEN).
- Multiplex PCR on the genes encoding the following *E. coli* virulence factors:
ST, LT, Eae, BfpA, VT1, VT2, EhxA and IpaH.
30   - Detection of PCR products by use of capillary electrophoresis
- This example describes a theoretical procedure for the above technologies.

Example 7:   -Subtyping of the virulence genes *vtx2* and *eae*, by direct sequencing of
             amplicons containing subtype distinguishable sequences.

Example 8:   - Optimization of critical parameters in the multiplex PCR.

5

Example 9:   - DNA purification from spiked stool samples by use of KingFisher and
             QIAamp DNA stool kit for the amplification in the multiplex PCR.

Example 1:

10
                              INTRODUCTION
The present example describes the use of multiplex-PCR in the identification of the *E. coli*
groups: ETEC, EPEC and A/EEC, VTEC and EIEC. The PCR relies on the specific
multiplex-amplification of the genes encoding the following virulence factors: heat-labile
enterotoxin (LT) characteristic for ETEC, heat-stable enterotoxin (ST) characteristic for
15   ETEC, intimin (Eae) characteristic for EPEC or VTEC, bundle-forming pilus (BfpA)
characteristic for EPEC, enterohemolysin (EhxA) characteristic for VTEC, vero toxin 1 and 2
(VT1 and VT2) characteristic for VTEC and invasive plasmid antigen (IpaH) characteristic
for EIEC. As a positive control for the PCR a primerset for the 16S rDNA gene from most
gram-positive bacteria was also included in the assay. The PCR analysis is performed on
20   template DNA derived from bacterial colonies grown on plates. Each PCR was analyzed by
agarose gel electrophoresis for the possible presence of amplicon(s) of the size that could be
identified as any of the virulence markers mentioned above. The present example shows how
the PCR method performs with respect to sensitivity and specificity. This includes the
analysis of serially diluted reference strain and analysis of a 124 reference strains. All
25   reference strains were also tested by a probe hybridization technique directed towards the
same genes.

                         MATERIALS AND METHODS
Membrane hybridisation:
30   Each virulence gene that were screened for were contained on a specific pBluescript clone.
These clones served as templates for the labeling reaction using the PCR DIG Labeling Mix
from Roche, and T3/T7 pBluescript primers or primers designed within the gene. Strains

used to construct the clones were obtained from The International *Esherichia* and *Klebiella* Centre, WHO, Statens Serum Institut, Denmark and genes encoding the following factors were included in the assay: Eae (8), VT1 (28), VT2 (26), STp/ST1A (24), STh/ST1B (24), LT (24) and IpaH (27).

5

By use of a 1µL sterile loop, small volumes of the different colonies, that were chosen to be investigated, were transferred to individual spots on a nylon membrane (Hybond-N$^+$, Amersham Biotech) that was positioned on top of an agar plate. After over night growth the nylon membrane was removed from the agar plate. The colonies on the nylon membrane

10   were lysed, denatured and neutralized by incubating the nylon membrane in the following solutions for 10 min each: 10% SDS, 0.5 N NaOH, 1.4 M NaCl/0.5 M NaOH, 1 M Tris-HCl, pH 7.4 and 1.5 M NaCl / 0.5 M Tris-HCl, pH 7.4. The membrane was then baked at 65°C for one hour and prehybridised in 0.1 x SSC and 0.1% SDS at 65°C.

15   The probe was denatured by boiling in hybridisation solution for 8 min (0.5% Blocking Reagent, 0.1% N-laurylsarcosine, 0.02% SDS and 5 x SSC). The pre-hybridisation solution was exchanged with the hybridisation solution containing the denatured probe and incubated at 65°C for one hour. The hybridisation solution was discharged and the membrane was washed twice in 2 x SSC, 0.1% SDS for 5 min at room temp, and twice in 0.1 x SSC, 0.1 %

20   SDS for 30 min at 65°C.

The hybridisation signals were developed by washing the membrane in Detection Buffer (0.1 M maleic acid, 0.15 M NaCl, 0.2 M NaOH, pH 7.5) for 2 min at room temperature, and in Blocking Buffer (1% Blocking Reagent, 0.1 M maleic acid, 0.15 M NaCl, 0.2 M NaOH, pH

25   7.5) at room temperature for 25 min. Next, 6µL Anti-digoxigenin was mixed with 60mL Blocking Buffer and added to the membrane and incubated at room temperature for 30 min. The membrane was then washed twice in Detection Buffer for 15 min each, and incubated for 2 min with Detection Buffer containing 50 mM MgCl$_2$.

Finally the membranes were incubated over night in the dark at room temperature in

30   Detection Buffer containing 50 mM MgCl$_2$, 0.15% NBT (4-Nitro blue tetrazolium chloride, Boehringer Mannheim) and 0.1% BCIP (x-phosphate/5-Bromo-4-chloro-3-indolyl-phosphate, Bohringer Mannheim). Next day, the colour reaction is stopped, by rinsing the

25

membrane in water, and the resulting hybridisation reactions were visualized for the individual spots.

PCR:

5   Relevant subtypes of each gene were downloaded from Gene Bank. Alignments (ClustalW algorithm) of gene sequences and primer design were done using the LaserGene Software DNAStar. Homologues of each gene that were included in the alignments are described above.

10   Primers sequences were chosen to have comparable GC content (45-60%), base pair length (20-24 base pairs) and melting temperatures (55-57°C), optimal 3' end and 5' end stability, and low likelihood of hairpin loop and primer-dimer formation.

Primer-sets based on the theoretical primer design, were tested experimentally at different
15   annealing temperature and different concentrations of PCR reagents. The resulting optimum conditions were taken into account when the primer sets were combined in the multiplex analysis. Primers were redesigned until they met the satisfactory level of sensitivity and specificity. Each primer set was chosen to result in a unique amplicon size that could be easily identified by agerose gel electrophoresis. See table 3 for primer sequences and
20   amplicon sizes.

Genes encoding the following virulence factors were included in the assay: heat-labile enterotoxin (LT) characteristic for ETEC, heat-stable enterotoxin (ST) characteristic for ETEC, intimin (Eae) characteristic for A/EEC, EPEC or VTEC, bundle-forming pilus (BfpA)
25   characteristic for EPEC, enterohemolysin (EhxA) characteristic for VTEC, vero toxin 1 and vero toxin 2 (VT1 and VT2) characteristic for VTEC, and invasive plasmid antigen (IpaH) characteristic for EIEC. As a positive control for the PCR, a primer set targeting 16S rDNA matching most gram-negative bacteria was also included.

PCRs of 25 µL were composed of the following reagents in the following order: 1x PCR
30   buffer (*Roche*), 260 µM of each of dATP, dCTP, dGTP, and 520 µM of dUTP (*GeneAmp, Applied Biosystems*), primermix (se table 3 for individual final concentration), 0.25 U

26

FastStart Taq DNA Polymerase (*Roche*), 0.25U UNG (*Applied Biosystems*), 2.6 mM MgCl₂ and 5 µL template DNA.

All 22 primer sequences and their individual final PCR concentrations are shown in table 3.

5   DNA amplifications were performed in a MJ Research (PTC-200) thermocycler, with the following program: 50 °C for 1 min, 94°C for 6 min, 35 cycles of [94°C for 50 s, 57°C for 40 s and 72°C for 50 s] and 72°C for 7 min.

Cell preparations

10   Bacterial templates for sensitivity studies were prepared as follows. Reference strain D2164, fr1368, D1826, D2168 and D2103 were grown to medium sized colonies (1-2 mm) on agarose plates. One of each colony was transferred to 100 µL 10 % Chelex (*Bio-rad*) and boiled for 5 min. The supernatants from the Chelex preparations were combined in the following way. Ten µL of strain fr1368 (*ipaH* positive) and 10 µL of strain D2164 (*vtx1*,

15   *vtx2*, *eae* and *ehxA* positive) were mixed and 10-fold serially diluted in water. Ten µL of each of these dilutions were mixed with 10 µL the D2103 (non-pathogenic strain) supernatant, from where 5 µL was used for PCR. Strains representing the other virulence genes D1826 (*eae* and *bfpA* positive) and D2168 (*sta* and *elt* positive) were prepared the same way. Bacterial templates for the specificity study, were prepared by growing reference strains on

20   agarose plates. One of each colony was transferred to 100 µL 10% Chelex (*Bio-rad*) and boiled. Five µL of the supernatant was used directly in the PCR.

Detection of PCR-products:

PCR products were identified on a standard agarose gel electrophoresis system by ethidium

25   bromide staining. Gels were made of 1.5% agarose and applied voltage was 4.5 volts/cm.

RESULTS AND DISCUSSION

PCR primers were designed on basis of the sequence comparisons described in section 5 (Detailed description of the invention). The amplicons representing the different virulence

30   genes were all of unique sizes, being easily distinguished by standard agarose gel electrophoresis (table 1). Intensive specificity and sensitivity studies are often not included in papers describing diagnostic PCR analyses (17,19) It is however, very important to test

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both parameters thoroughly before an experimental procedure is introduced into a diagnostic laboratory.

Table 4. Strains used to test the sensitivity limit of the multiplex-PCR assay. Strain fr1368 and strain D2164 were mixed in equal volumes and 10-fold serially diluted relative to the non-pathogenic strain D2103, thereby testing the sensitivity limit of the *ipaH, vt1, vt2, eae* and *ehxA* genes. Strain D2168 and stain D1826 were treated likewise in order to test the sensitivity of *sta, elt, eae* and *bfpA* genes.

Strain nr.	Virulence gene(s)
fr1368	*ipaH*
D2164	*vxt1, vtx2, eae, ehxA*
D2168	*sta, elt*
D1826	*eae, bfpA*
D2103	non-pathogenic strains, no virulence genes

During development and optimising of the present PCR setup, a number of reference strains were tested in 10 fold serial dilutions (table 4). In order to mimic a more realistic situation 2 dilutions were made; one contains *ipaH, vtx1, vtx2, eae* and *ehxA* (figure 1), and another dilution contains *sta, elt, eae* and *bfpA* (figure 2). Besides the 10-fold dilutions, the pathogenic strains were also diluted in a constant volume of a non-pathogenic *E. coli* strain, mimicking a situation where very few pathogenic bacteria are present in a population of non-pathogenic bacteria. This is probably the most important way to test the sensitivity limit of PCR assays, as this allows the assay to be applied on a mixture of colonies grown from faecal sample of diarrheagenic patients. Moreover, this PCR could be applied on total DNA extracted from faeces, even if very few pathogenic bacteria are present. For both dilutions, specific amplicons were detected until a dilution of $1/10^4$ relative to D2103. If one medium sized colony is estimated to contain 10^8 bacteria and there is 1/20 colony present in each PCR, this means that at a pathogenic strain dilution of $1/10^4$ this would correspond to a sensitivity limit at approximately 500 bacteria per PCR.

In order to test the specificity of the assay 124 reference strains obtained from The International *Esherichia* and *Klebiella* Centre, WHO, Statens Serum Institut, Denmark, were tested by the assay.

The strains were collected over a period of 19 years (1984 to 2003), and are therefore expected to constitute a broad range of relevant clinical samples representing many different homologues of the different genes (table 5). All PCR results showed the same virulence

5   genes as also identified by standard DNA hybridisation. Example of PCR results are shown in figure 3 and 4.

10   **Table 5.** 124 reference strains obtained from the International *Esherichia* and *Klebiella* Centre, WHO, Statens Serum Institut, Denmark. Before PCR, all strains were serotyped and tested for the 8 virulence genes by DNA hybridisation. When tested by the multiplex-PCR method, all strains were found positive for the exact same virulence genes as was found by hybridisation. DAEC and EAggEC were identified by probe hybridisation for other genes not included in the PCR method.

Nr.	E. coli Group / Shigella	Year	Serotype	sta	vtx1	bfpA	eae	vtx2	elt	ehxA	ipaH	Negative for the 8 virulence genes
1	A/EEC	2003	O4, 123:H-				+					
2	A/EEC	2003	O145:H-				+			+		
3	A/EEC	2003	O116:H+				+					
4	A/EEC	2002	Orough:H8				+					
5	A/EEC	2002	O118				+					
6	A/EEC	2002	O35,135:H11				+					
7	A/EEC	2002	O51:H49				+					
8	A/EEC	2002	Orough:H33				+					
9	A/EEC	2003	O177:H25				+			+		
10	A/EEC	2003	O132:H34				+					
10	DAEC	1999	O21:H10									+
11	DAEC	2000	O15:H-									+
12	DAEC	2000	O21:K-:H11									+
13	DAEC	2000	O36:H4									+
14	EAggEC	2001	O107:H+									+
15	EAggEC	2001	O153:H2									+
16	EAggEC	2001	O92:H33									+
17	EAggEC	2001	O103:H+									+
18	EAggEC	2001	O92:H+									+
19	EAggEC	2001	O150:H28									+
20	EAggEC	2001	O24:H+									+
21	EAggEC	2001	O49:H-				+			+		
22	EAggEC	2001	O113:H-									
23	EIEC	2000	O64:H-								+	
24	EIEC	2000	O64:H-								+	
25	EIEC	2001	O+:H-								+	
26	EIEC	2001	O121:H-								+	
27	EIEC	2001	O28ac:H-								+	
28	EIEC	2001	O173:H-								+	
29	EIEC	2002	O144:H-								+	
30	EIEC	2001	O124:H30								+	
31	EIEC	2002	O143:H-								+	
32	EIEC	2002	O173:H-								+	

29

No	Type	Year	Serotype	1	2	3	4	5	6	7
33	EIEC	2002	O+:H-							+
34	EIEC	2002	O28ac:H-							+
35	EPEC	2002	O103:H2			+				
36	EPEC	2002	O111:H-		+	+				
37	EPEC	2002	O 26:H-			+			+	
38	EPEC	2002	O142:H34		+	+				
39	EPEC	2002	O129:H11			+				
40	EPEC	2002	O111:H38			+				
41	EPEC	2002	O111:H9			+				
42	EPEC	2002	O114:H49			+				
43	EPEC	2002	O145:H34			+				
44	EPEC	2002	O121:H19			+				
45	EPEC	2002	O126:H6			+				
46	EPEC	2002	O 26:H -			+			+	
47	EPEC	2002	O 55:H 7			+				
48	EPEC	2002	O125ab:H 5			+				
49	EPEC	2002	O127:H-		+	+				
50	EPEC	2002	O145:H-			+			+	
51	EPEC	2003	O 26:H-			+				
52	EPEC	2003	O145:H-			+			+	
53	EPEC	2003	O157:H7			+				
54	EPEC	2003	O114:H-			+				
55	EPEC	2003	O86:H8			+				
56	ETEC	2000	Orough:H-							+
57	ETEC	2001	Orough:H-	+				+		
58	ETEC	2000	O167:Hru					+		
59	ETEC	2000	O 6:H16	+				+		
60	ETEC	1996	O115:K?:H5	+						
61	ETEC	1996	O8:K+:H9	+						
62	ETEC	1998	Orough:H-					+		
63	ETEC	2003	O4							+
64	ETEC	1996	O78:K-H11	+				+		
65	ETEC	2002	O148	+				+		
66	ETEC	2000	O8:H9	+				+		
67	ETEC	2000	O39:H12	+				+		
68	ETEC	2000	O128ac:H+	+				+		
69	ETEC	2000	O17:Hrough	+				+		
70	ETEC	2001	O169:H-					+		
71	ETEC	2001	O56:H-					+		
72	ETEC	2000	O8:H9					+		
73	VTEC	2002	O157:H-		+	+	+		+	
74	VTEC	2002	O157:H-		+	+	+		+	
75	VTEC	2002	O157:H-		+	+	+		+	
76	VTEC	2002	O157:H-		+	+	·+		+	
77	VTEC	2002	O157:H-		+	+	+		+	
78	VTEC	1994	O157:K:H-		+	+			+	
79	VTEC	1997	O157:H 7		+	+			+	
80	VTEC	1998	O157:H 7		+	+	+		+	
81	VTEC	1999	O157:H-		+	+			+	
82	VTEC	2002	O157:H 7			+	+		+	
83	VTEC	2002	O157:H 7			+	+		+	
84	VTEC	2002	O157:H 7			+	+		+	
85	VTEC	2002	O157:H 7			+	+		+	
86	VTEC	2002	O157:H-			+	+		+	
87	VTEC	2002	O103:H 2		+	+			+	

30

88	VTEC	2002	O103:Hru	+	+		+	
89	VTEC	2002	O103:H 2	+	+		+	
90	VTEC	2002	O103:H 2	+	+		+	
91	VTEC	2002	O103:H 2	+	+		+	
92	VTEC	1999	O103:H 2		+	+	+	
93	VTEC	1999	O103:H 2		+	+	+	
94	VTEC	1999	O103:H 2		+	+	+	
95	VTEC	2001	O 26:H-	+	+	+	+	
96	VTEC	1984	O 26:H11		+	+	+	
97	VTEC	2001	O 26:H-	+	+	+	+	
98	VTEC	2002	O 26:H11	+	+	+	+	
99	VTEC	2002	O 26:H11	+	+		+	
100	VTEC	2002	O 26:H11	+	+		+	
101	VTEC	2002	O 26:H11	+	+		+	
102	VTEC	2002	O 26:H11	+	+		+	
103	VTEC	2002	O 26:H11	+	+		+	
104	VTEC	2000	O 26:H11		+	+		
105	VTEC	2002	O 26:H11		+	+	+	
106	VTEC	2002	O 26:H-		+	+	+	
107	VTEC	2002	O 26:H11		+	+	+	
108	VTEC	2001	O145:H+	+	+		+	
109	VTEC	2001	O145:H+	+	+		+	
110	VTEC	2001	O145:H+	+	+		+	
111	VTEC	2001	O145:H28	+	+		+	
112	VTEC	2002	O145:H28	+	+		+	
113	VTEC	2002	O145:H-		+	+	+	
114	VTEC	2002	O145:H-		+	+	+	
115	VTEC	2002	O145:H-		+	+	+	
116	VTEC	2002	O145:H-		+	+	+	
117	VTEC	2002	O145:H -		+	+	+	
118	*Shigella*	2001	*sonnei*					+
119	*Shigella*	2001	*flexneri*					+
120	*Shigella*	2001	*fleneri* 1b					+
121	*Shigella*	2001	*flexneri* 2a					+
122	*Shigella*	2001	*boydii* 1-7					+
123	*Shigella*	2001	*dysenteriae* 2-7					+
124	*Shigella*	2001	non-agglutinable (2)					+

Finally, 16 non-*E. coli* strains were tested by the PCR method in order to investigate any cross reaction to other species. The strains were: *Salmonella enterritidis, Salmonella para A, Salmonella typhimurium, Vibrio cholera, Aeromonas caviae, Shigella flexneri 2 a, Shigella dysenteri 3, Proteus, Pseudomonas, Plesiomonas shigelloides, Serratia marcescens, Shigella sonnii, Klebsiella, Citrobacter freundii, Salmonella cholerasuis, Yersinia* ent. biotype5, 27. Except for the 3 *Shigella* species that showed an *ipaH* band, non of the other species resulted in any signals (data not shown)

Example 2:

In this examples a less complex multiplex-PCR assay was tested on 499 of clinical samples that were also tested by DNA hybridisation. This multiplex-PCR is able to identify the same *E. coli* groups (VTEC, EPEC and A/EEC, ETEC or EIEC) as the method described in

5   examples 1, but relies on fewer genes and therefore gives a less informative diagnostic answer. The PCR analysis is directed towards genes encoding the following virulence factors: heat-labile enterotoxin (LT) characteristic for ETEC, heat-stable enterotoxin (ST) characteristic for ETEC, intimin (Eae) characteristic for EPEC, A/EEC or VTEC, vero toxin 1 and 2 (VT1 and VT2) characteristic for VTEC and invasive plasmid antigen (IpaH)

10   characteristic for EIEC. The PCR analysis is performed on template DNA derived from bacterial colonies grown from faecal samples. Each PCR was analysed by agarose gel electrophoresis for the possible presence of amplicon(s) of the size that could be identified as any of the virulence markers mentioned above. In this example, the diagnostic quality of multiplex-PCR analysis is compared to membrane hybridisation, which is a traditional

15   method for diagnostics of pathogenic *E. coli* groups.

                                MATERIALS AND METHODS

Sample handling:

Bacterial colonies were cultured from faecal samples as follows. A small volume

20   (approximately 0.1 g) of the faeces sample was gently shaken in 2 mL sterile buffered saltwater (80 mM NaCl, 50 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 7.38). Approximately 10 µL of that suspension was streaked out onto an agar plate (*SSI Enteric Medium, Statens Serum Institut, Denmark*) and grown overnight at 37°C.

25   Membrane hybridisation:

As described in Example 1.

Template preparation:

From each clinical samples a number of morphological different colonies were picked from

30   the plate grown cells and transferred to the same 200 µL 10 % Chelex (*Bio-Rad*) and boiled for 5 min. Five µL of supernatants were used for PCR.

32

PCR

The multiplex-PCR contained primer set for the following genes: e*lt*, *sta*, *eae*, *vtx1*, *vtx2*, *ipaH* and *16S* rDNA in concentration listed in table 3. All other parameters and reagents were the same as described in example 1.

Detection of PCR-products:

PCR products were identified on a standard agarose gel electrophoresis system by ethidium bromide staining. Gels were made of 1.5% agarose and applied voltage was 4.5 volts/cm.

RESULTS AND DISCUSSION

Growing faecal samples on SSI Enteric Media allows a certain differentiation of bacterial species based on phenotypical characteristics. Different strains are however not always associated with a significant phenotype. It is therefore important to pick as many different colonies as possible from a plate, in order to increase the chance of having the possible pathogenic bacteria included. With the sensitivity limit established in example 1, a positive result can be obtained if just one of the picked colonies among 10^4 colonies is positive.

Table 6. Performance of multiplex-PCR and membrane hybridisation in faeces diagnostics of *E. coli*. A total of 499 samples were analysed; both method found the same 27 samples positive and 465 samples negative. 5 samples were tested positive only by PCR, whereas 2 samples were positive only by hybridisation. All discrepancies were double tested by PCR.

Gene(s)	Found by both methods	Only found by multiplex-PCR	Only found by DNA-hybridisation	Total
Negative	465			
eae	19	2	1	
vtx1	1	1		
vtx2	1	1		
elt	3			
vtx1 +eae	1			
ipaH	2	1	1	
Total	492	5	2	499

In the present example 499 clinical samples were tested by both multiplex-PCR and DNA hybridisation. The results are summarized in table 6. Both methods detected the same 465 samples as negative and 27 positive samples distributed on 6 genotypes. Five samples were found positive only by PCR, whereas 2 samples were positive only by DNA hybridisation.

5   All discrepancies were retested by PCR and showed the same result, decreasing the likelihood of a failed PCR. One explanation of the different result could therefore be differences in the quality of the picked colonies. As different persons might have picked different colonies and as some plates might have had very few pathogenic colonies on them, some differences are expected to be present in the DNA composition used in the 2 assays.

10   With the relative higher number of positives found by PCR and the obvious time saving advantages, the PCR method is concluded to be superior to the hybridisation technique.

Example 3:

15   INTRODUCTION

Multiplex-PCR analysis for the diagnosis of the pathogenic *E. coli* strains; VTEC, EPEC and A/EEC, ETEC or EIEC in human faecal samples. The PCR analysis is performed on template DNA purified directly from human stool samples. The subsequent PCR analysis is directed towards genes encoding the following virulence factors: LT characteristic for ETEC, ST

20   characteristic for ETEC, Eae characteristic for EPEC, A/EEC or VTEC, BfpA characteristic for EPEC, EhxA characteristic for VTEC, VT1 and VT2 characteristic for VTEC and IpaH characteristic for EIEC. Completed PCRs were analysed by the Luminex® technology, where PCR products are hybridised to specific capture-probes situated on microbeads.

25   MATERIALS AND METHODS

DNA preparation:

DNA isolation from faecal samples was done by using the KingFisher 96™ from ThermoLabsystem, according to the manufactures instruction. Briefly, a small volume (approximately 0.1 g) of the faeces sample was gently shaken in 2 mL sterile buffered

30   saltwater (80 mM NaCl, 50 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 7.38). 200 µL of each faecal suspension was mixed with 750 µL lysis buffer and loaded in separate wells in a 96 well microtiter plate. Other plates were prepared containing either: washing buffer, 70% ethanol,

distilled water and suspensions of magnetic particles. Five μL of the final DNA concentrate was used per PCR.

Sensitivity studies were performed, by adding 10-fold serially dilutions, of strains harbouring virulence gene(s), to a faecal sample tested negative for that specific gene(s).

5

PCR:

PCR conditions were the same as described in Example 1, except that the forward primers were 5'-biotinylated in this analysis.

10   Detection of PCR-products:

PCR products were detected by the Luminex® technology. Amplicons were labelled by having the forward primers 5'-biotinylated. Capture probes (listed in table 7) were synthesized with a 5'-amine Uni-Link modification, for the coupling to the carboxylated microbeads.

15

Each probe was coupled to a uniquely coloured population of carboxylated microbeads. This was done by mixing 1 nmol of amine-substituted probe with a suspension of 5×10^6 microbeads in 50 μL 0.1 M 2-(N-Morpholino) ethanesulfonic acid, pH 4.5 [MES], followed by addition of 25 μg N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide [EDC] and

20   incubation in the dark for 30 min. The EDC addition and incubation were repeated and the microbeads were washed once with 0.02% Tween-20 and once with 0.1% SDS. Coupled microbeads were stored in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) in the refrigerator in the dark until hybridization.

Five μL of the PCRs was denatured at 95°C for 10 min and added to the hybridization

25   solution (3 M tretramethylammonium chloride, 50 mM Tris-HCl, pH 8.0, 4 mM EDTA, pH 8.0, 0.1% sakrosyl) containing a mixture of 5000 of each probe-coupled microbead in a 50 μL total reaction volume. Reactions were hybridized at 55°C for 10 min, pelleted by microcentrifugation and resuspended in 50 μL hybridization solution. Hybridized amplicons were labelled with 120 ng streptavidin-R-phycoerythrin at 55°C for 5 min (62 μL total

30   reaction volume). Reactions were then analyzed on the Luminex® 100.

Table 7. Oligonucleotide sequence of the capture probes used in the hybridization analyses of the 9 genes. Probe design is described in "Detailed description of the invention".

Virulence factor	Encoded by gene	Oligonucleotide probe sequences (5'→ 3')
Heat labile entero-toxin I (LT)	*elt*	CTGGATTCATCATGCACCACAAGGTTGTG
Heat stable entero-toxin I (ST)	*sta*	CACAGCAGTAATTGCTACTATTCATGCTTTCAGGA
Heat stable entero-toxin I (ST) human	*estA-human*	GTCCTGAAAGCATGAATAGTAGCAA
Heat stable entero-toxin I (ST) porcine	*estA-porcin*	GAGACTAAAAAGTGTGATGTTGTAAA
Intimin (Eae)	*eae*	TACCCGTTTAGGTATTGGTGGCGAATACTGG
Verocytotoxin 1 (VT1)	*vtx1*	TCCAGAGGAAGGGCGGTTTAATAATCTACGG
Verocytotoxin 2 (VT2)	*vtx2*	TGGTTTCATCATATCTGGCGTTAATGGAGTTCAG
Invasion plasmid antigen H (IpaH)	*ipaH*	CCAGCATCTCATACTTCTGCTCTTCTGCCTG
Enterohemolysin A (EhxA)	*ehxA*	TGCTGAGAAAACAACGGGAAGGAGAGGA
Bundle-forming pilus A (BfpA)	*bfpA*	TCAGAAGTAATGAGCGCAACGTCTGCAATT
16S rDNA	*16S*	AACGTATTCACCGTGGCATTCTGATCCAC

5   DISCUSSION

Primer- and probe design were performed as described in Example 1 and in "Detailed description of the invention". The different probes directed towards the gene encoding ST can be combined in any combination preferred. For example, both *estA-human* and *estA-procine* can be immobilized on the same bead if no distinction between the two variants are

10   preferred, or on separate beads if information on subtype variants is desired.

KingFisher™ is an semi-automated equipment that can purify DNA from complex biological materials in about 30 minutes. The technique relies on the binding of DNA to magnetic particles that are efficiently separated and washed to produce a pure template DNA

15   preparation. The apparatus handles 96 samples in one analytical setup by use of microtiter plates. Four microtiter plates containing different reagents need to be manually prepared before hand. A number protocols have all ready been established for the preparation of template DNA from different starting materials. These include for example blood, different tissues and stool samples. With this flexibility in mind, the KingFisher™ is expected to

20   perform well on most chemical complex primary sources.

Compared to growing samples, DNA purification directly from the primary source has several advantages. One, the time of analysis is reduced, as this automated DNA purification can be done in less than as hour, compared to the over night growth needed to obtain usable plate grown colonies. Two, DNA purified directly from the source should represent the

5　different DNA populations in the same relative distribution as in the sample, including DNA from dead bacteria. This is advantageous since *in vitro* growth will be favorable to some bacteria and make others loose their plasmids. Three, the automated procedure using this apparatus requires less hands-on-time than the growing of samples, which also reduces the tedious and repetitive manually work.

10

When the Luminex® technology is applied for the detection of PCR products, the analysis is based on a stringent hybridization to specific capture probes. Each specific capture probes is situated on a population of microbeads that has a distinguishable internal colour. The hybridization reaction is automatically analyzed by measuring the combination of microbead-

15　colour and the PCR product colour-labeling, one bead at a time. Due to limitations of the internal color-coding of the microbeads, the technology has a maximum potential of screening with 100 probes at a time. One hundred gene probes are expected to go a long way, even if other diarrheagenic bacteria like for example *Salmonella, Camphylobacter, Yersinia* and *Klebsiella*, and genes encoding antibiotic resistance are included in the assay. In this

20　case, the multiplex-PCRs need to be divided into a suitable number of reactions. The Luminex technology has proven very sensitive and able to discriminate between single mutaions in SNP analysis (29), and is therefore expected to enclose the analytical capacity necessary for the present diagnostic assay.

Compared to gel electrophoresis the Luminex technology has a number of advantages: 1) the

25　sequence specific hybridization adds more specificity to the diagnostic assay, compared to the un-precise size determination of gel electrophoresis, 2) the Luminex procedure is faster, semi-automated and therefore requires less hands-on-time 3) expansion of gene number would be limited in gel electrophoresis as each gene has to have a distinguishable size, whereas Luminex will screen for up to 100 genes in the same reaction.

30

Example 4:

INTRODUCTION

Multiplex-PCR analysis for the diagnosis of the diarrheagenic *E. coli* strains; VTEC, EPEC
and A/EEC, ETEC or EIEC in human faecal samples. The PCR analysis is performed on
5   template DNA purified directly from human stool samples, by use of BUGS'n BEADS ™
from Genepoint A.S. (Oslo, Norway). The subsequent PCR analysis is directed towards
genes encoding the following virulence factors: LT characteristic for ETEC, ST characteristic
for ETEC, Eae characteristic for EPEC, A/EEC or VTEC, BfpA characteristic for EPEC,
EhxA characteristic for VTEC, VT1 and VT2 characteristic for VTEC and IpaH
10   characteristic for EIEC. Completed PCRs were analyzed by the hybridization to specific
capture probes that were immobilized on plastic surfaces (PCR-ELISA) and microarrays.

MATERIALS AND METHODS

15   <u>DNA preparation:</u>
A small volume (approximately 0.1 g) of the faeces sample was gently shaken in 2 mL sterile
buffered saltwater (40 mM NaCl, 25 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 7.38). This
suspension was left standing for 2 min to obtain a supernatant free from most undissolved
materials. The DNA was isolated by use of the BUGS'n BEADS ™ from Genepoint A.S.
20   (Oslo, Norway). 500 µL supernatant for the sample was mixed with 400 µL Binding Buffer
and 20 µL Bacterial Binding Beads in a 1.5 mL microcentrifuge tube. The bead/bacterial
complex was held on the inside of the tube, by using an external magnet, while the
supernatant was poured off. The bead/bacterial complex was dissolved in 50 µL Lysis Buffer
and incubated at 80°C. After 5 min 150 µL 96% cold ethanol was added and the incubation
25   was continued for another 5 min. The bead/bacterial complex was retained by magnetic force
while the supernatant was discharged. The bead/bacterial complex was washed twice with
70% ethanol. The bead/bacterial complex was finally resuspended in 30 µL H_2O, from where
5 µL was used for PCR.

30   Probes for both ELISA hybridization and microarrays are the same. Probe- and primer design
was based on the criteria describes in "Detailed description of the invention".

ELISA hybridisation

The attachment of the capture probes to the solid surface and the hybridizations were done with NucleoLink™ Surface (PCR-ELISA) from Nalge Nunc International according to the manufactures instructions. Oligonucleotide probes for each of the 9 genes are listed in table

5   7. Each probe was synthesized with 12 T's added to the 5-end, and the final T in the 5'-end being phosphorylated. The 12 T's served as a linker that increases the hybridization efficiency by lifting the probe away from the solid surface. The phosphorylation was necessary for the covalent binding of the probe to the plastic surface. Approximately 100 nM of each of the capture probes were used in the covalent binding to the surface of separate

10   wells in a 96-well microtiter plate. The PCR was performed as described in Example 1 except that primers were biotinylated at the 5'-end and the total reaction volume was 100 µL. The hybridization was done by adding 10 µL of the PCR to each of the 9 wells containing the different capture probes. After a standard hybridization procedure, including NaOH denaturation, hybridization at 52°C for 2 h and SSC incubations and washing, the reaction

15   was detected by use of Alkaline phosphatase conjugated streptavidin (DAKO, Denmark).

Microarray detection

Primers used in the PCR were Cy3-labelled in the 5'-end, and the total reaction volume was 100 µL. All other PCR parameters were the same as described in Example 1. Completed

20   PCRs were purified with the QIAquick PCR Purification kit from QIAGEN, dried and resuspended in 4 µL H$_2$O, 7.5 µL 20 x SSC, 2.5 µL 1% SDS, 1 µL salmon sperm DNA (10 mg/mL) and 15 µL formamide. This suspension was denatured at 94°C for 5 min prior to the hybridisation.

25   Capture probes used in this experiment are listed in table 7. Approximately 3 nmol of each probes was dissolved in 20 µL 6 N Na$_2$SCN. One nL of the probes were arrayed onto coated glass slides (Amersham Pharmacia Biotech) and baked at 80°C for 2 h. Prior to pre-hybridization the slides were washed in isopropanol and boiling water and dried in ultra clean N$_2$. The pre-hybridization consisted of 20 min incubation at 60°C in 3.5 x SSC, 0.2% SDS

30   and 1% BSA, rinsing in water and isopropanol and drying in ultra clean N$_2$ gas. Thirty µL of the denatured sample was added to the pre-hybridized slide and covered with a coverslip and stored in a moisturized hybridization chamber for 14 h at 35°C. The coverslip was removed and the slide was washed 5 times in SSC and 0.1% SDS, SSC sequentially being 2x, 1x, 0.1x,

0.1x and 0.1x. The slides were finally dried in ultra clean N_2. The hybridization signal was analysed by a confocal laser microscope (Molecular Dynamics, California).

DISCUSSION

5   Bugs n' Beads™ is a simple and relative inexpensive procedure for the preparation of bacterial DNA from growth media and biological samples. It is designed, first to separate bacteria by their adherence to magnetic beads, and then to prepare DNA from the isolated bacteria. The bacterial-bead extraction is a powerful preparation technique, as is separates bacteria from all non-bacterial materials in one single step. Also, if the cell count of the

10   sample is low, this concentrating procedure will help to obtain a detectable signal. The technique is therefore expected to perform well on chemical complex samples as for example faeces or foods. The relative simple procedure of Bugs n' Beads™ is well suited for automatisation, which would be valuable for a routine diagnostic laboratory.

15   ELISA hybridization is a relative established method, which therefore has the advantages of being less prone to technical problems. Each gene must be hybridized in separate tubes/wells, which makes the procedure more tedious than Luminex, where all hybridizations can take place in the same reaction tube.

20   Microarrays has the advantage of being extremely well suited for screening of many genes in the same sample. This has been shown usable in many studies dealing with for examples the expression of thousands of genes. In the case of analyzing many samples for the existence of a few genes, microarrays are relative expensive. The technique is therefore expected to be most relevant when a more complete assay is undertaken, dealing with many different strains,

25   antibiotic resistance and subtyping. With the proper design, such a "universal pathogen microarray" would find use in many places and eventually on the commercial marked at a lower price.

Compared to gel electrophoresis, both ELISA and microarrays have the following
30   advantages 1) the sequence specific hybridization adds more specificity to a diagnostic assay compared to the un-precise size determination of gel electrophoresis, 2) the Luminex procedure is faster, semi-automated and therefore requires less hands-on-time 3) expansion of

gene number would be limited in gel electrophoresis as each gene has to have a
distinguishable size, whereas Luminex will screen for up to 100 genes in the same reaction.

Example 5

INTRODUCTION

Multiplex-PCR analysis for the diagnosis of the pathogenic *E. coli* strains; VTEC, EPEC and
A/EEC, ETEC or EIEC in human faecal samples. The real-time PCR analysis was performed
on template DNA purified directly from human stool samples by use of FTA Technology
from Whatman. Genes encoding the following virulence factors were targeted: LT
characteristic for ETEC, ST characteristic for ETEC, Eae characteristic for EPEC, A/EEC or
VTEC, BfpA characteristic for EPEC, EhxA characteristic for VTEC, VT1 and VT2
characteristic for VTEC and IpaH characteristic for EIEC.

MATERIALS AND METHODS

DNA preparation:

The DNA extraction was performed by FTA® Technology from Whatman. A small volume
(approximately 0.1 g) of the faeces sample was gently shaken in 2 mL sterile buffered
saltwater (40 mM NaCl, 25 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 7.38). This suspension was
left standing for 2 min to obtain a supernatant free from most undissolved materials. Ten µL
of that supernatant was transferred to the FTA Card and allowed to dry. A small circle of two
mm in diameter of the card containing the sample was punched out and put into a 0.5 mL
PCR tube. The punch was first washed three times in 200 µL FTA Purification Reagent and
then washed twice in TE. After the final wash the punch was allowed to air dry and used
directly as template in the PCR amplification.

Real-time PCR:

Due to fluorescence overlap and technical equipment limitations, real-time multiplex PCR is
presently limited to analyze four genes in the same reaction. Therefore, the total analysis of 9
genes needed to be performed in three separate multiplex reactions. The combinations of
genes, primers, probes, fluorophores and quenchers in each of the three reactions are listed in
table 8. The primers used in this analysis are the same as in the previous examples (see table

41

3 for primer sequences and table 7 for probe sequences). In each reaction the different probes
were coupled to fluorophores with distinguishable emission spectra, and a compatible
quenchers. For analysis of *sta* both *estA*-human (alternatively *sta*) and *estA*-porcine probes
will be labelled with the same fluorochrome, and both primer pairs will be present in the

5 same reaction. This will not allow discrimination between the two gene variants (humane vs
porcine) but identify any of the two variants present in a giben sample. The analysis was
performed on a Mx4000™ Multiplex Quantitative PCR System from Stratagene.

Table 8. Real time PCR parameters. See table 2 and 5 for primer and probe sequences.

Reaction number	Genes targeted	Forward / reverse primer	Forward / reverse primer concentration (μM)	Probe labelling: 5'-fluorophore / 3'-quencher	Probe concentration (μM)
1	*sta*	ST-F / ST-R	0.3 / 0.3	5-FAM / TAMRA	0.2
	vtx1	VT1-F / VT1-R	0.25 / 0.25	5-ROX / BHQ-2	0.15
	ehxA	Ehx-F / Ehx-R	0.15 / 0.15	Cy5 / BHQ-3	0.2
	16S	16S-F / 16S-R	0.25 / 0.25	HEX / BHQ-1	0.1
2	*elt*	LT-F / LT-R	0.4 / 0.4	5-FAM / TAMRA	0.15
	eae	Eae-F / Eae-R	0.2 / 0.2	5-ROX / BHQ-2	0.2
	ipaH	IpaH-F / IpaH-R	0.1 / 0.1	Cy5 / BHQ-3	0.2
	16S	16S-F / 16-R	0.25 / 0.25	HEX / BHQ-1	0.1
3	*bfpA*	Bfp-F / Bfp-R	0.2 / 0.2	5-FAM / TAMRA	0.2
	vtx2	VT2-F / VT2-R	0.25 / 0.25	5-ROX / BHQ-2	0.15
	16S	16S-F / 16-R	0.25 / 0.25	Cy5 / BHQ-3	0.15

10 BHQ: balck hole quencher

Each PCR parameter was tested at different values in order to find optimum condition that
resulted in highest sensitivity and specificity of every gene in the assay. The total reaction
volume was 25 μL and contained the following reagents: 1x PCR buffer (Applied

15 Biosystems), 260 μM of each of dATP, dCTP, dGTP and 520μM og dUTP (Applied
Biosystems), primer- and probe- combinations and concentrations as listed in table xx. 0.5U
AmpliTaq Gold® DNA Polymerase (Applied Biosystems), 0.25U UNG (Applied
Biosystems), 3.2 mM MgCl$_2$ and 5 μL template. The temperature parameters were as
follows: 2 min at 50°C, 10 min at 94°C and 40 cycles of 15 sec at 95°C and 1 min at 58°C.

20 Data analysis was done by the software included in the Mx4000™ Multiplex Quantitative
PCR System.

42

DISCUSSION

FTA technology from Whatman is based on cell lysis and DNA entrapment in the fibrous matrix of a specialized membrane. In the subsequent washing steps the cell debris and PCR inhibitors are removed. Finally, a small piece of the membrane is added directly to the PCR from where the DNA still bound to the matrix serves as a template for the amplification reaction. The FTA technology is expected to perform well in the DNA purification from many different starting materials, including stool samples. This is based on the fact, that the DNA is bound relative stable early in the purification procedure to the membrane, and that this allows an intense washing of the DNA-membrane complex, resulting in an efficient removal of PCR inhibitors.

Primers and probes are designed as described in "detailed description of the project" and in Example 1 and 3. The real-time-multiplex analysis is restricted to four simultaneous reactions per reaction tube because of the overlap in emission spectra. However, as the 9-gene multiplex PCR analysis has been optimized to work well under non real-time PCR conditions, it is believed that when the technology is developed to contain more fluorophores in the same reaction, it should be possible to use the present PCR setup in a "9-gene-multiplex- real-time-PCR" setup. Until then, the 9-gene analysis is divided into 3 reaction-tubes reaction, which is still an reasonable analytical strategy.

Example 6

INTRODUCTION

Multiplex-PCR analysis for the diagnosis of the pathogenic *E. coli* strains; VTEC, EPEC, ETEC or EIEC in human faecal samples. The PCR analysis is performed on template DNA purified directly from human stool samples by using the QIAamp® DNA Stool Mini Kit (QIAGEN). The subsequent PCR analysis is directed towards genes encoding the following virulence factors: LT characteristic for ETEC, ST characteristic for ETEC, Eae characteristic for EPEC, A/EEC or VTEC, BfpA characteristic for EPEC, EhxA characteristic for VTEC, VT1 and VT2 characteristic for VTEC and IpaH characteristic for EIEC. Completed PCRs were analysed by capillary electrophoresis.

Materials and methods:

<u>DNA preparation:</u>

The DNA was purified by QIAamp® DNA Stool Mini Kit from QIAGEN according to the provided instructions. Briefly, 0.1-0.2 g of faces was measured into a 2 mL microcentrifuge

5   tube and mixed with 1.4 mL Buffer ASL. This suspension was incubated at 70°C for 5 min, vortexed for 15 s and centrifuged for 1 min. The supernatant was then treated with InhibitEX that binds PCR inhibitors and precipitates them during a 3 min of centrifugation step at 13.000 g. 600 μL of the supernatant was then treated with 25 μL Proteinase K, mixed with Buffer AL, vortexed for 15 s and incubated at 70°C for 10 min. The lysate was mixed equal

10   volumes of 96% ethanol spun in QIAamp spin columns. The bound DNA was washed by first running 500 μL Buffer AW1 and secondly, by running 500 μL Buffer AW2 through the column. The DNA was eluted by incubating the column with 200 μL Buffer AE for 1 min and spinning the column at 13.000g for 1 min.

15   <u>PCR:</u>

PCR conditions were the same as described in Example 1, except that the forward primers were synthesized containing 5-FAM on the 5'-prime end.

<u>Detection of PCR-products:</u>

20   The PCR products were analyzed by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) according to the manufactures instructions. The capillary was 47 cm by 50 μm, the temperature was constant at 60°C, and the electrical field was 15 kV. One microliters of the completed PCRs and 1 μL 2500-TAMRA labelled size standard were denatured by incubating in 12 μL formamide at 95°C for 3 min, and cooled on ice before

25   loading on the analyser. The resulting peaks (5-FAM labelled) were identified by comparison to the size standard.

DISCUSSION

30

QIAamp® DNA Stool Mini Kit from QIAGEN is developed to purify DNA directly from stool samples, either with increased ratio of non-human to human DNA or vise versa

depending on the analytical need. The kit relies on bacterial lysis, specific absorption and precipitation of PCR inhibitors and DNA binding to washable centrifuge columns. If the centrifugation step is exchanged with a vacuum manifold, the whole procedure can be automated making it ideal for a routine laboratory.

5

Capillary electrophoresis is seen as a fast and more precise alternative to gel electrophoresis. The time saving depends on sample capacity of the apparatus and the number of samples to be analyzed. If hundreds of samples are to be analyzed the gel electrophoresis might be faster if big gels with high sample number capacity are used. When is comes to size determination,

10   capillary electrophoresis is clearly superior to gel electrophoresis, offering an overall more specific assay.

Example 7

15   INTRODUCTION

This examples deals with the subtyping of the *E. coli* virulence genes: *vtx1*, *vtx2* and *eae*. The severity of the clinical manifestations of *vtx2* and/or *eae* infections, are believed to be at least partly determined by the specific subtype (5). The specific subtype should be taken into

20   consideration when the proper treatment is determined. Subtyping of these important virulence markers, is also valuable for epidemiological surveillance. Based on sequence data, the *eae* genes can be divided into a least 7 subtypes, whereas more than 6 subtypes of *vtx2* have all ready been identified. Many subtyping studies are based on the detection of fragment patterns of PCR products being digested by restriction enzymes (1,2,25). Unfortunately, such

25   methods are based on relative few sequence characteristics, and restricted to include sequence spots to where suitable restriction enzymes can be found. Also, these methods can be misleading, if a new mutation occurs at a site where the enzyme was designed to cut. Direct sequencing of PCR product by standard sequencing reaction, will easily yield 500 readable base pairs, which if chosen right can be very discriminating between different

30   subtypes. As the cost of sequencing reactions, have been much reduced over the last couple of years, this method is now feasible for routine diagnostic analyses.

Example 8:

INTRODUCTION

Optimisation of multiplex-PCR analysis designed for the diagnosis of the diarrhoeagenic *E.*
5   *coli*; VTEC, EPEC and A/EEC, ETEC and EIEC. Stable and reproducible analyses are of
outmost importance for routine diagnostic analyses. Therefore, each critical parameter of the
analysis was intensively tested, in order to obtain the analysis with the best possible
performance.

MATERIALS AND METHODS

10

Template DNA preparation

Bacterial templates were prepared as follows: Reference strain D2164 (*eae, vtx1   vtx2* and
ehxA positive), fr1368 (*ipaH* positive), D2168 (*sta* and *elt* positive) and D2103 (non-
pathogenic *E. coli* strain) were grown to medium sized colonies (1-2 mm) on agarose plates.
15   One of each colony was transferred to 100 µL 10 % Chelex (*Bio-rad*) and boiled for 5 min.
Supernatants were used directly in PCR, after they were combined in the following way:
Strain D2164 was diluted 0, 5x, 10x and 20x in strain D2103. Strain fr1368 and D2168 were
mixed in equal volumes and then diluted 0, 5x, 10x and 20x in strain D2103. Five µL of each
dilution were added as template in the PCRs. The resulting 8 bacterial dilutions were tested at
20   each concentration of the variable PCR reagents.

PCR

PCR was preformed as described in example 1. One parameter was examined at the time, by
changing it to different values, while all others were kept constant (table 9). Each PCR was
25   run on a standard agarose gel electrophoresis system by ethidium bromide staining. Gels
were made of 1.5% agarose and applied voltage was 4.5 volts/cm.

RESULTS AND DISCUSSION

30   Six critical parameters in the multiplex PCR were tested at different concentrations, on DNA
templates, derived form different pathogenic reference stains in different dilutions relative to
a non-pathogenic reference strain. This was done to obtain the most stable and reproducible

analysis, and to identify the chemical composition of the PCR that resulted in the analysis
with the highest sensitivity and highest signal to noise ratio. It was found that the method had
a relative wide functional interval for most of the parameters. This is important for routine
laboratories, as small daily differences must not influence the analytical outcome. The

5   optimal value reflected the best performance with respect to sensitivity and signal to noise
ratio, and was positioned central in the functional interval.

Table 9. Tested PCR parameters on different dilutions of different positive *E. coli* strains. Functional interval

10   refers to PCR composition where all expected bands were visible at good intensity. Optimal value refers to the
PCR composition that resulted in the best amplification of the expected products. 1) See materials and methods
for preparation. 2) See materials and methods and table 3 for composition.

PCR parameter	Functional interval	Optimal value
Template DNA [1]	2-8 µL	5 µL
PCR buffer	0.8 – 1.5x	1.2x
dNTP	200 – 260 µM	240 µM
Primer mix [2]	2.0 – 4.5 µL = 0.6 x – 1.3x	3.5 µL = 1x Primermix
Polymerase	1.2 – 1.3 U	1.25 U
$MgCl_2$	3.6 – 4.0 mM	4.0 mM

15   **Example 9:**

INTRODUCTION

Multiplex-PCR analysis designed for the diagnosis of the diarrhoeagenic *E. coli*; VTEC,
EPEC and A/EEC, ETEC and EIEC, performed on DNA derived directly from faecal

20   samples. Diagnosis of pathogenic bacteria in human faeces without a growth step before
PCR, is an attractive strategy for many reasons. This will save the time consuming growth
step of at least one day, and has the potential of detecting dead bacteria in the sample, and
bacteria prone to loose plasmid-bound virulence factors. The present examples describes the
performance of two commercial kit used for the preparation of template DNA from spiked

25   faecal samples.

MATERIALS AND METHODS

2 bloody and 2 non-bloody stool samples (negative for pathogenic *E. coli*) where chosen for the spiking experiment. Liquid pathogenic *E. coli* cultures were added to the stools, resulting

5 in final concentrations of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 bacteria per mL stool of either a VTEC strain (*eae, vtx1* and *vtx2* positive) or an ETEC strain (*elt* and *staI* positive) and an EIEC strain (*ipaH* positive). Template DNA for the PCR was extracted from the stools by either KingFisher™ mL (*Thermo Labsystems*) or QIAamp® DNA Stool Kit (*Qiagen*), according the manufactures instructions. The PCR was performed as described above using

10 one µL of the eluate from KingFisher™ mL and 5 µL of the eluate from QIAamp® DNA Stool Kit.

RESULTS AND DISCUSSION

15 DNA was purified directly from spiked faecal samples, and subsequently analysed by the multiplex PCR method. Liquid cultures were used to construct spiked faecal samples, containing $10^2 - 10^8$ pathogenic *E. coli* per mL stool. *E. coli* negative stools samples, were either combined with a VTEC strains containing *eae, vtx1* and *vtx2*, or an EIEC strain containing *ipaH* and an ETEC strain containing *staI* and *elt*, each in the final concentration

20 described above. For each strains combination two bloody and two non-bloody stool samples were extracted by either KingFisher (*Thermo Labsystems*) or QIAamp® DNA Stool Kit (*Qiagen*) and subjected to the multiplex PCR. Both commercial kits are based on DNA liberation in bacterial lysis buffer, and DNA extraction by binding to immobilised silica. KingFisher is a relative inexpensive robot equipment offering a 15 minutes automated DNA

25 purification once reagents and samples are manually prepared for the procedure. QIAamp® DNA Stool Kit is a manual kit that requires about 45 minutes for the preparation of 24 samples. Automatisation is possible, but requires a more complicated robot equipment. Each diagnostic locus could be identified at 10^6 cells per mL stool, for both extraction procedures, except one bloody stool extracted with KingFisher, from where no DNA could be amplified

30 (table 10). DNA preparations that has inhibitory effect on PCR amplification, is determined by the failure to amplify the 16S DNA positive control. Such samples need to be reanalysed or analysed in a different way.

48

Table 10. Sensitivity limits for the multiplex-PCR method on pure cultures prepared by simples boiling and spiked faecal samples extracted by KingFisher (*Thermo Labsystems*) and QIAamp (*Qiagen*). *) one bloody stool sample could not be amplified after KingFisher extraction.

Starting material	Template DNA preparation method	Sensitivity limit
Pure cultures	simple boiling	~ 10^3 cells per PCR
Bloody or non-bloody	KingFisher™ mL (*Thermo Labsystems*)	10^6 cells per mL stool
spiked stools *	QIAamp® DNA Stool Kit (*Qiagen*)	10^6 cells per mL stool

5

As for both the KingFisher™ mL and QIAamp® DNA Stool Kit procedures, the DNA was eluted in the same volume as the faecal volume entering the extraction procedure. Thus, even with 100% DNA recovery during the extraction procedure, 10^6 cells per mL stool would yield 10^6 cells per mL eluate. Given that 1 or 5 µL of the eluate was used in PCRs, 10^6 cells

10 per mL stool equals 10^3 or $5 * 10^3$ cells per PCR, which is comparable to the sensitivity limit of the DNA extraction from pure cultures (see table 10). Hence, except the one bloody stool extracted by KingFisher, both methods perform well with respect to the removal of faecal PCR inhibitors. Different PCR conditions were tested for an increased sensitivity, but neither the addition of BSA, changes in eluate volume per PCR, or cycle number had any effect.

15

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